Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: B. Singh, P.M. Sahu, S.C. Jain & S. Singh (2002) Antineoplastic and Antiviral Screening of Pyrrolizidine Alkaloids from Heliotropium subulatum, Pharmaceutical Biology, 40:8, 581-586, DOI: 10.1076/phbi.40.8.581.14659

To link to this article: https://doi.org/10.1076/phbi.40.8.581.14659

Published online: 29 Sep 2008.

Pharmaceutical Biology
2002, Vol. 40, No. 8, pp. 581–586

1388-0209/02/4008-581$16.00

Antineoplastic and Antiviral Screening of Pyrrolizidine Alkaloids from *Heliotropium subulatum*

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Abstract

The phytochemical investigation of *Heliotropium subulatum*, guided by bioassay, led to the isolation of heliotrine, 7-angeloyl heliotrine, retronecine, subulacine and subulacine-*N*-oxide. It has been reported that 7-angeloyl heliotrine was the major alkaloid both *in vivo* (0.42%) and *in vitro* (0.25%) cell cultures. Antineoplastic, cytotoxic and antiviral activities of ethanol, hexane, dichloromethane crude extracts and isolated pyrrolizidine alkaloids have been screened by packed cell volume and plaque inhibition methods. The 7-angeloyl heliotrine and retronecine showed activity at 5 µg/kg/day of 41.7 and 38.6% inhibition against Sarcoma 180. The hexane extract (3 µg/ml) and 7-angeloyl heliotrine (10 and 5 µg/ml) showed selective cytotoxicity against Chinese hamster V_{79} cells. The ethanol and hexane crude extract showed significant antiviral activity to *Coxsackie, Poliomyelitis* and *Measles* at 500 and 100 µg/ml concentrations, while heliotrine and 7-angeloyl heliotrine possess activity against *Poliomyelitis* and *Vesicular stomatitis* at a concentration of 10 µg/ml.

Keywords: Pyrrolizidine alkaloids, *Heliotropium subulatum*, cytotoxic activity, antitumor, antiviral, callus culture.

Introduction

Heliotropium subulatum Hochst. ex DC. (syn. *H. zeylanicum* Clarke; Boraginaceae) is an annual herb and distributed in arid zone parts of Rajasthan. Its ethanol extract exhibited anticancer activity against human epidermoid carcinoma of nasopharynx *in vitro* cell cultures as well as P-388 lymphocytic leukemia in mice and also showed hypoglycaemic effects. It was devoid of antiprotozoal, CNS, CVS, antispasmodic and diuretic effects (Jain & Defilipps, 1991; Aswal et al. 1984). Preliminary studies with *Heliotropium* species demonstrated pyrrolizidine alkaloids – heliotrine, europine (Jain & Singh, 1998; Jain & Purohit, 1986), heliotridine, lasiocarpine (Reina et al., 1995), subulacine, subulacine-*N*-oxide (Malik & Rehman, 1988). These alkaloids possess antitumor property including N-256, P-388, L-1210, P-1534 and melanoma B-16 (Pandey et al., 1983) and antimicrobial (Jain & Singh, 1998). However, monocrotaline, spectabilline and senecionine reported to be active against Walker-256 (Marquina et al., 1989). Besides these reports, we have also investigated some triterpenoids from this plant species (Jain & Singh, 1999).

As part of a systematic survey of botanical sources for antineoplastic and antiviral activity, the ethanol, hexane, dichloromethane crude extracts and pyrrolizidine alkaloids of the aerial parts of *H. subulatum* were examined and gave reproducible activity against Sarcoma 180, Chinese hamster V_{79} cells and selected viruses. However, there are no reports in the literature on isolation and characterization of pyrrolizidine alkaloids from this plant species both *in vivo* and *in vitro* cell cultures and their anticancer, antiviral screening. Hence, the current study was carried out.

Materials and methods

Plant material

H. subulatum was collected (July–August, 1997) from the fields of the Agricultural Research Station, Durgapura, Jaipur and authenticated from the Herbarium, Department of Botany, University of Rajasthan, Jaipur, India (sheet no. 19439).

Accepted: April 30, 2002

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Extraction and initial separation of alkaloids

Plant material was defatted with petroleum ether (5.0 L; 60–80 °C) for 24 h, filtered and the residue was Soxhlet extracted with 95% ethanol (v/v) for 36 h, filtered and evaporated to dryness (yield; 2.68%). The crude ethanol extract was subjected to partition between hexane (Fr. I) and dichloromethane (Fr. II). It yielded a hexane eluate (98.68 g), which was used in subsequent work. The column chromatography of Fr. I on TLC grade silica gel (E-Merck) with eluation by chloroform – methanol; 8 fractions, A-H, were collected.

Heliotrine (1) and 7-angeloyl heliotrine (2) – A portion of fraction A and B (14.31 g) was crystallized from methanol. The purification of crystalline material by preparative TLC, with development by chloroform – methanol – ammonia; 85 : 14 : 1, R_f ~ 0.35 followed by final crystallization from methanol, yielded **1** (279 mg), mp 128 °C [(Leonard, 1960) mp 128 °C]. The fraction C (8.91 g) was subjected to column chromatography on TLC grade silica gel, with elution by benzene-chloroform (8 : 2) to yield a major fraction, this fraction gave pure **2** (109 mg) on crystallization from methanol. Both alkaloids were identical with the samples previously described (Warren, 1970).

Retronecine (3) – Fraction D, E (16.41) was subjected to preparative TLC with ethylacetate-acetone-ethanol – ammonia (5 : 3 : 1 : 1; v/v) with two developments. The UV absorbing band was scraped and eluted to yield 643 mg of fraction, which was crystallized from methanol, to yield **3** (225 mg), mp 121–122 °C [(Bull et al., 1968) mp 121–122 °C]. The spectroscopic properties (IR, NMR, UV, MS) of isolated material were consistent with this structural assignment.

Subulacine (4) and subulacine-N-Oxide (5) – Fraction F and G were combined (18.21 g) and subjected to chromatography on silica gel with elution by chloroform-methanol (85 : 15) yielded 2 fractions. Purification of fraction 1 (3.21 g) by preparative TLC, with development by chloroform – methanol – ammonia (85 : 14 : 1), yielded 312 mg, its IR spectrum was superimposable to that of authentic samples. Fraction H (11.67 g) was subjected to preparative TLC in chloroform – methanol – ammonia (85 : 14 : 1). The material had an R_f ~ 0.45 in above mentioned solvent system, crystallized from methanol, yielded 217 mg, identical with the authentic samples previously described (Malik & Rehman, 1988), which were supplied by Malik and Rehman, University of Karanchi, Pakistan.

Tissue culture

Unorganised callus tissue of *H. subulatum* was raised by seedlings. Seeds were surface sterilized with 0.1% $HgCl_2$ (w/v) solution for 2–3 min and then rinsed 3 times with sterilized distilled water. The seeds were then aseptically inoculated onto Murashige and Skoog (1962) medium supplemented with 1.0–5.0 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5–2.5 ppm benzyl amino purine (BAP) and 3.0% sugar. The seeds took 10–15 days to germinate and seedlings thus formed started differentiated tissue formation after 20–25 days of inoculation. The cultures were incubated at 25 ± 1 °C with 60% relative humidity under room light conditions (300 Lux). The callus tissue was transferred onto fresh medium after 4–5 weeks interval. The callus tissue was harvested at the age of 2, 4, 6, 8 and 10 weeks and growth index was calculated (GI = Final dry weight of tissue – Initial dry weight of tissue/Initial dry weight of tissue).

The fresh tissue samples (300 g) were first dried at 100 °C for 15 min to inactivate any enzymatic activity and then at 40 °C until the weight became constant. The each of the dried tissue sample was powdered, refluxed in a Soxhlet apparatus with 95% ethanol for 36 h, filtered and evaporated to dryness (yield; 2.65, 3.13, 3.42, 3.62 and 3.02%). These extracts (callus tissue) were dried *in vacuo*. The crude ethanol extract of callus and aerial parts was subjected to partition between hexane and dichloromethane. The hexane eluate of each sample was used for quantification of pyrrolizidine alkaloids, by using HPLC, Millipore Waters Model 501, fitted with pump delivery system, injector (Model 6 UK) with solvent delivery system (Millipore Waters, Millford, MA, USA) by using μ Bondapak C_{18} column (30 cm × 3.9 mm; 24 ± 1 °C temperature), mobile phase methanol (HPLC grade; E-Merck), flow rate 1.0 ml/min, effluent attenuation at 0.5 AUFS (Lambda Max Model 481, spectrophotometer), absorbance adjusted to 254 nm and chart speed 0.5 cm/min.

Animals

Antineoplastic activity was performed with ICR albino mice weighing 20–25 g each, 5-weeks-old, housed in an air conditioned room and fed a standard laboratory diet and tap water throughout the experiment.

Antineoplastic screening

Total packed cell volume method (Itokawa et al., 1979) was adopted for antineoplastic screening. Sarcoma 180 (1×10^{-6} cells/0.1 ml ascitic fluid) was implanted (i.p.). Each group of experiment contained 6 animals. The test sample was suspended in physiological saline solution was mixed with (0.5%) carboxymethyl cellulose (CMC) and injected at the concentration of 50, 100 μg/kg/day of test crude extracts, while 5,10 μg/kg/day of test compounds (i.p.) for consecutive 5 days. The control set of animals received saline only. Each of the experimental animal was sacrificed after 7 days from the implantation of ascite tumor. The ascites of each animal was centrifuged (3000 rpm) for 5 min to isolate the tumor cells. The volume of tumor cells (packed cell volume: PCV) and total volume of tumor cells (total volume: TV) were determined in each case. The body weight change was also recorded by determining the difference in the weight after 7 days of ascite tumor implantation and the weight of tumor implanted. The inhibition (%) was calculated as the

ratio between the average PCV of control – average PCV of treated/average PCV of control.

Cytotoxicity screening

Using cloned Chinese hamster V_{79} (supplied by Dr. Tsukagoshi, JFCR) which were maintained on RPMI-1640 medium (Nizshipharm Co. Ltd.), supplemented with 10% fetal calf serum (Mitsubishi Chem Co.) and kanamycin (100 µg/ml), cytotoxicity was tested. The cells (3×10^5 cell/well) were cultured in a corning disposable 6 cells plate containing 2 ml of growth medium/cell incubated at 30 °C, in humidified atmosphere of 5% CO_2. Varied test drug concentrations (10 µl) were added to cultures at day 1 after the transplantation. The colonies were fixed with 10% formaldehyde solution (2 ml; 20 min) and stained with 0.05% crystal violet (0.75 ml) at day 5. The cytotoxicity of test drug was assessed by determining the number of stained colonies of test group/those of control group × 100 values in the drug containing medium relative to colony growth (0.5%) medium at 5 days after the drug treatment.

Antiviral screening

Poliomyelitis type 1 strain $1A/S_3$ (Van Den Berghe et al., 1978) and *Measles* Edmonston A were plaque purified, while *Herpes* type 1 and *Vesicular stomatitis* were isolated by Dr. S. Pattyn, University of Antwerp, Belgium. However, *Coxsackie* B_2 virus from NIH, Bethesda, USA and *Semliki forest* L. supplied by Dr. C.J. Brandis, Porton Down, Salisbury, England. *Coxsackie, Poliomyelitis* and *Semliki forest* virus stocks were prepared in Hella suspension cells, whereas, *Herpes, Measles, Vesicular stomatitis* were grown in VERO cells. The culture medium was supplemented (Hronovsky et al., 1975) with 2% new born calf serum, penicillin G (100 units/ml), streptomycin (0.5 mg/ml), pH 7.2 with 1 N NaOH. The viral titers of *Herpes* and *Semliki forest* viruses were estimated by determining limiting dilutions, which could initiate infection in 50% of cultures inoculated. The 50% end points were calculated following an established protocol (Reed & Muench, 1938).

The viral titers of *Poliomyelitis* (10^8 PFU/ml; plaque forming units), *Coxsackie* (10^7 PFU/ml), *Measles* and *Vesicular stomatitis* (10^6 PFU/ml), *Semliki forest* (10^7 TCD_{50}/ml; tissue culture infective dose) and *Herpes* (10^5 TCD_{50}/ml) viruses were estimated by the plaque formation method on VERO monolayer cells (Dulbecco, 1952).

Each of the crude extracts and pure samples of alkaloids were dissolved in sterile 0.01 M physiological Tris buffer (8 ml; pH 7.2), diluted with tissue culture medium (M-2; 128 ml; pH 7.7). These test samples were filtered through Whatman GF-2 paper disc followed by sterilization (Millipore filter, 0.02 µm) and stored at 30 °C.

The antiviral bioassay of certain fractions and pure pyrrolizidine alkaloids was confirmed by the plaque inhibition method (Dulbecco, 1952). The confluent cultures of VERO cells were prepared in tissue culture dishes (50 mm diameter) and infected with 0.2 ml *Poliomyelitis* virus suspension medium containing 100 PFU. After a 1 h absorption period, the cells were washed to remove any unabsorbed virus overlaid with tissue culture medium and 0.8% acetone purified agar at 42 °C. After solidification of paper discs (6 mm diameter), impregnated with 0.2 ml serial of two-fold dilutions, of fractions and pure compounds in culture medium were centrally placed on surface of agar and incubated at 37 °C for 2 days. A second agar overlay on the same composition supplemented with 0.03% neutral red was added and incubated at 37 °C for 2 days and later plaque inhibition zones were recorded.

Results and discussion

The aerial parts of *H. subulatum* were extracted with ethanol and the crude ethanol extract, after partitioning, gave 98.68 g (hexane eluate) containing a number of pyrrolizidine alkaloids. The compounds were isolated from the fractions (A-H) by column chromatography on silica gel, followed by preparative tlc. On the basis of spectral data and by direct comparison with authentic samples, compounds were identified as heliotrine (**1**; 279 mg), 7-angeloyl heliotrine (**2**; 109 mg), retronecine (**3**; 643 mg), subulacine (**4**; 312 mg) and subulacine-N-oxide (**5**; 217 mg).

Table 1 lists the isolated pyrrolizidine alkaloids from *in vitro* cell cultures. Observed results indicated that the growth index (0.69; 2 weeks < 1.95; 4 weeks < 3.42; 6 weeks < 5.61; 8 weeks > 5.09; 10 weeks) was higher at 8 week old callus and later starts to decrease in growth. In this report, the 7-angelgoyl heliotrine was found to be maximum in quantity both *in vivo* (0.42%) and *in vitro* (0.25%). The quantity of pyrrolizidine alkaloids increased from 2 to 8 week old callus while after 8 weeks their amounts decreased in quantity up to 10 weeks. These results supported the view that the growth of callus and the quantity of alkaloids interrelated to biosynthetic potentiality of plant. These alkaloids were quantified by HPLC and identified by their retention times (El-Dahmy & Ghani, 1995) which were identical to their standards.

The antineoplastic activity of ethanol, hexane, dichloromethane crude fractions and the isolated alkaloids as heliotrine, 7-angeloyl heliotrine, retronecine, subulacine and subulacine-*N*-oxide were investigated and it was found that 7-angeloyl heliotrine exhibited maximum inhibition (41.7%) at the concentration of 5 µg/kg/day (Table 2). It is noteworthy that retronecine, subulacine-*N*-oxide and ethanol extract (100 µg/kg/day) possess antineoplastic activity at a concentration of 5 µg/kg/day.

The cytotoxicity of the pyrrolizidine alkaloids heliotrine, 7-angeloyl heliotrine, retronecine, subulacine and subulacine-*N*-oxide are presented in Table 3. The Chinese hamster V_{79} *in vitro* assay system appears to be more sensitive to most alkaloids tested than does the antineoplastic assay, and it thus might prove to be a generally useful screen for compounds of this

Table 1. Quantification of pyrrolizidine alkaloids from *H. subulatum* both *in vivo* and *in vitro*.

isolated compounds	% of isolated compounds					
		in vitro[1]				
	in vivo	2	4	6	8	10
heliotrine	0.21	0.004	0.017	0.028	0.033	0.031
7-angeloyl heliotrine	0.42	0.012	0.031	0.061	0.079	0.071
retronecine	0.16	0.009	0.026	0.039	0.043	0.041
subulacine	0.29	0.008	0.014	0.026	0.036	0.039
subulacine-*N*-oxide	0.32	0.010	0.019	0.033	0.040	0.037

[1] Age of callus in weeks.

Table 2. Antineoplastic activity of certain fractions and pyrrolizidine alkaloids from *H. subulatum* against Sarcoma 180.

fractions or alkaloids	dose µg/kg/day	BWC[1]	PCV[2] T/C	inhibition[3] (%)
ethanol extract	50	−1.51	0.25	19.3
	100	−1.39	0.21	32.2
hexane fraction	50	−1.13	0.24	22.5
	100	−1.18	0.26	16.1
dichloromethane fraction	50	+2.41	0.28	09.6
	100	+1.98	0.29	06.4
heliotrine	5	+1.11	0.23	25.8
	10	+1.41	0.25	19.3
7-angeloyl heliotrine	5	−1.09	0.19	41.7
	10	−1.21	0.20	35.4
retronecine	5	−1.16	0.18	38.6
	10	−1.23	0.24	19.3
subulacine	5	−1.44	0.24	19.3
	10	−1.35	0.26	16.1
subulacine-N-oxide	5	−1.28	0.21	30.2
	10	−1.31	0.20	25.4

[1] Body weight change = weight of ascite tumor − weight of tumor.
[2] Packed cell volume/treated/control.
[3] Inhibition % = PCV of control − PCV of treated/PCV of control.

Table 3. Cytotoxic activity of certain fractions and pyrrolizidine alkaloids from *H. subulatum* against Chinese hamster V_{79} cells.

fractions or alkaloids	T/C (%) number of colonies[1]							
	concentration = µg/ml							
	100	50	30	20	10	5	3	1
ethanol extract	50.6	69.1	72.8	79.0	81.4	85.1	75.3	64.1
hexane fraction	59.2	70.3	55.5	53.1	54.3	45.6	40.7	56.7
dichloromethane fraction	51.8	64.1	69.1	71.5	67.9	75.3	81.3	79.0
heliotrine	77.7	79.0	64.1	51.8	72.8	46.9	48.1	61.7
7-angeloyl heliotrine	74.0	65.4	66.9	43.0	41.9	42.4	60.4	50.6
retronecine	83.9	76.5	80.2	69.4	66.6	77.7	59.2	75.3
subulacine	80.2	58.0	56.7	70.3	44.4	46.9	65.4	53.0
subulacine-*N*-oxide	72.9	60.4	59.2	62.9	55.9	45.6	50.6	48.3

[1] An activity of T/C (%) < 53.4% considered as active.

Table 4. Virucidal activity of certain fractions and pyrrolizidine alkaloids from *H. subulatum*.

fractions or alkaloids	dose µg/ml	antiviral activity[1]					
		Poliomyelitis	*Coxsackie*	*Semliki forest*	*Vesicular stomatitis*	*Herpes simplex*	*Measles*
ethanol extract	100	10	10	1	10	1	1
	500	100	1000	1	100	10	1
hexane fraction	50	100	10	1	1	1	100
	100	1000	100	10	10	1	1000
dichloromethane fraction	50	1	1	10	1	1	1
	100	10	10	10	10	1	1
heliotrine	5	100	10	1	100	10	1
	10	1000	100	10	1000	100	10
7-angeloyl heliotrine	5	1	100	10	100	1	100
	10	10	1000	100	1000	10	1000
retronecine	5	1	1	1	1	1	1
	10	1	10	10	10	1	1
subulacine	5	1	1	100	1	1	1
	10	10	10	1000	10	1	1
subulacine-N-oxide	5	100	100	1	1	1	1
	10	1000	1000	1	1	1	10

[1] Antiviral activity expressed as the reduction factor of the viral titer.

type. The observed results indicated that the hexane fraction, showed potent cytotoxic activity at a concentration 3 µg/ml, while 7-angeloyl heliotrine possessed cytotoxic activity at concentrations of 10 and 5 µg/ml, respectively. The extracts of unsaturated pyrrolizidine alkaloids possess cytotoxic *in vitro* activity to Ehrlich ascites carcinoma cells (Wassel et al., 1987).

The ethanol and hexane crude extracts showed significant antiviral activity to *Coxsackie, Poliomyelitis* and *Measles* at concentrations of 500 and 100 µg/ml, respectively, while among pyrrolizidine alkaloids, heliotrine and 7-angeloyl heliotrine possessed activity (1×10^2 to 1×10^3 reduction factor) to *Poliomyelitis*, *Vesicular stomatitis*, *Coxsackie* and *Measles* at a concentration of 10 µg/ml. It is noteworthy that subulacine and subulacine-*N*-oxide also showed potent activity at 10 µg/ml, concentration dependently (Table 4). The retronecine and dichloromethane extract did not exhibited any activity with the selected viruses.

Acknowledgements

We wish to thank to Dr. H. Itokawa, College of Pharmacy, Tokyo, for his kind help with the antineoplastic and cytotoxic testing, Professor A.J. Vlientinck, University of Antwerp, Belgium, for carrying out the antiviral testing of the extracts and pure compounds, and the Department of Biotechnology, New Delhi, for financial support.

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